HSP90 supports tumor growth and angiogenesis through PRKD2 protein stabilization

Ninel Azoitei[1], Kristina Diepold[1], Cornelia Brunner[2], Arefeh Rouhi[3], Felicitas Genze[4], Alexander Becher[1], Hans Kestler[5], Johan van Lint[6], Gabriela Chiosis[7], John Koren III[7] Stefan Fröhling[8], Claudia Scholl[3], Thomas Seufferlein[1]

Authors' Affiliations
[1]Center for Internal Medicine I, University of Ulm, 89081 Ulm, Germany
[2]Institute for Physiological Chemistry, University of Ulm, 89081 Ulm, Germany
[3]Center for Internal Medicine III, University of Ulm, 89081 Ulm, Germany
[4]Department of Urology, University of Ulm, 89081 Ulm, Germany
[5]Institute for Neuroinformatic, Ulm University, 89081 Ulm, Germany
[6]Department of Molecular Cell Biology, Katholieke Universiteit, B-3000 Leuven, Belgium
[7]Department of Molecular Pharmacology and Chemistry, Memorial Sloan-Kettering Institute, New York, NY 10065
[8]Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, 69120 Heidelberg, Germany

Corresponding Author:
Ninel Azoitei
Center for Internal Medicine I, University of Ulm
Albert-Einstein-Allee 23, 89081-Ulm, Germany
ninel.azoitei@uni-ulm.de
Phone: +49-731-500 45726
Fax: +49-731-500 44665

Running title: PRKD2 modulates HSP90 tumor growth and angiogenesis

Keywords: PRKD2, HSP90, HIF-1α, hypoxia, tumor angiogenesis

Conflicts of interest: Memorial Sloan-Kettering Cancer Center holds the intellectual rights to PU-H71. Samus Therapeutics, of which G. Chiosis has partial ownership, has licensed PU-H71.

Abstract

In various types of malignancies, conventional therapies such as surgery, radiation and chemotherapy are often ineffective. The identification of dependencies on signaling proteins and pathways specific to cancer cells, has resulted in the development of new therapies with enhanced selectivity and efficacy and reduced toxicity. We have recently shown that PRKD2 is a crucial regulator of tumor cell – endothelial cell communication in gastrointestinal tumors and demonstrated the kinase to be a regulator of glioblastoma growth. Here we report that HSP90/CDC37 chaperone complex binds to and stabilizes PRKD2 in human cancer cells. Pharmacologic inhibition of HSP90, using structurally divergent small molecules currently in clinical development, induced proteasome-mediated degradation of PRKD2 and was associated with augmented apoptosis in human cancer cells of various tissue origin. Ectopic expression of PRKD2 protected cancer cells from the apoptotic effects of HSP90 abrogation and restored blood vessel formation in two animal models. We further demonstrate that PRKD2 is essential for hypoxia-induced HIF-1α accumulation and NF-κB activation in tumor cells. Moreover, overexpression of PRKD2 was able to partially restore HIF-1α protein levels and secreted VEGF-A in hypoxic cancer cells treated with HSP90 inhibitors. Taken together, our findings indicate that signals from hypoxia and HSP90 pathways are connected and further funneled by PRKD2 through the NF-κB / VEGF-A axis to promote tumor growth and tumor angiogenesis.

Introduction

Cancer development is a multistep process characterized by a multitude of genetic and epigenetic changes that induce resistance to pro-apoptotic stimuli, sustain angiogenesis and confer insensitivity to antigrowth signals and immune surveillance (1).

Rapid tumor growth often results in hypoxia, which triggers the stabilization of the transcription factor hypoxia-inducible factor-1 (HIF-1), an oxygen sensor that controls the expression of multiple target genes implicated in angiogenesis, metabolism and cell survival (2, 3). A prominent target of HIF-1α is the vascular endothelial growth factor A (VEGF-A) which induces tumor angiogenesis by stimulating proliferation, survival and migration of endothelial cells (4). HIF-1α has been reported to physically interact with heat shock protein 90 (HSP90) (5, 6) which can be targeted by small molecule inhibitors of chaperone, a growing class of clinically utilized anti-tumorigenic agents. HSP90 is a highly conserved and ubiquitously expressed molecular chaperone involved in the correct folding and final maturation of a plethora of proteins, so-called HSP90 clients, in an effort to maintain cellular homeostasis (7, 8). There are more than 200 HSP90 clients known including protein kinases, transcription factors and steroid hormone receptors (9 - 11). HSP90 is recruited to its kinase clients through interactions with kinase-specific co-chaperone CDC37 (12, 13), which stabilizes the HSP90/kinase (14). In tumor cells, HSP90 aids in folding dysregulated oncoproteins helping to sustain their aberrant activity. Amongst the most known client kinases of HSP90 are SRC (15), AKT (16), PDK-1 (17) and PKC (18). The latter was shown to directly activate protein kinase D (PRKD) family members via phosphorylation at two critical serine residues within the activation loop of the kinase catalytic domain (19). Recently, an affinity-based proteomic screen conducted to identify cancer-specific networks coordinated by HSP90 revealed PRKD2 as a potential client for the chaperone in chronic myeloid leukemia (CML) cells (20). The serine-threonine kinase PRKD2 and its sister isoforms PRKD1 and PRKD3 belong to the calcium/calmodulin-dependent protein kinase superfamily (21) and are activated by various stimuli including phorbol esters, reactive oxygen species (ROS), receptor tyrosine kinases and hypoxia (22 - 24). PRKD2 expression and activity correlates positively

with the state of de-differentiation in lymphoma (25) and was demonstrated to be involved in myeloid leukemia by activating NF-κB transcription factors (26). Furthermore, PRKD2 is involved in migration, invasion and growth of glioblastoma and pancreatic cancer cells (27 - 29). We have recently identified PRKD2 as a crucial mediator of hypoxia-induced VEGF-A expression and secretion in pancreatic cancer cells (24).

The aim of this study was to interrogate the contribution of PRKD2 to HSP90-mediated tumor growth and tumor angiogenesis. In addition, the involvement of PRKD2 in the regulation of hypoxia-mediated HIF-1α stabilization, NF-κB activation and VEGF-A production in the context of pharmacologic inhibition of HSP90 represented a major focus of our work. We identified PRKD2 as a novel client of HSP90 and revealed its requirement for tumor viability and tumor angiogenesis during abrogation of chaperone activity *in vitro* and *in vivo*. The fact that HSP90 regulates the stability of PRKD2, which acts as a two-pronged protein mediating tumor blood vessel formation via hypoxia-induced HIF-1α stabilization, VEGF-A production and tumor vascularization on the one hand and cancer cell viability on the other, makes HSP90 inhibition a strategy to target two cancer characteristics with one drug. Our work shows that PRKD2 represents a “station/convergence/nodal point” that appears not only integrate but also to funnel/further transmit hypoxia and chaperone’s molecular signals to activate a crucial family of transcription factors, namely the NF-κB and their target gene VEGF.

In conclusion, our data indicate that HSP90 inhibitors (PU-H71 and STA-9090) currently undergoing clinical evaluation in patients might be used to target cancer growth and blood vessel formation particularly in hypoxic tumors with high expression of PRKD2. Given current efforts to develop PRKD2 kinase inhibitors, we envision the combined use of HSP90 and PRKD2 kinase inhibitors to achieve synergistic effects.

Material and Methods

For details, see Supplementary Data.

Cell lines and inhibitors

Cancer cell lines originating from ATCC were cultured in early passages in DMEM (Invitrogen, Germany) supplemented with 10% fetal calf serum (FCS: PAA, Germany), 1% penicillin/streptomycin and 5 µg/ml Plasmocin Prophylactic (InvivoGen). HCT-116 colon cancer cells were maintained in McCoy media supplemented with 10% FCS. MG-132 was obtained from Sigma-Aldrich, bortezomib was purchased from LC Laboratories. PU-H71 was synthesized as reported (30). STA-9090 was purchased from SelleckChem.

Plasmids, transfection and lentiviral transduction

The Block-IT Pol II miR RNAi sequences (#NM_016457.3-1295 and #NM_016457.3-1295-2019, Invitrogen) targeting human PRKD2 cloned into pLenti6.4CMV/R4R2/V5-DEST vector via Gateway technology (Invitrogen) used for lentiviral mediated knock-down were described previously (24). The pLenti6.2-V5-DEST-PRKD2 overexpression vector was generated using the pDONR-223-PRKD2 entry clone from Addgene (#23490). High-titer virus-containing supernatants of 293FT after transient co-transfection of lentiviral vectors with pMD2 and psPAX2 viruses were used for lentiviral mediated transduction of cancer cells.

CAM assay

HCT-116 or MDA-MB231 cancer cells (1 x 10^6) were xenografted within 5 mm silicon rings on the surface of the chorionallantoic membrane (CAM) 8 days after fertilization. HSP90 inhibitor was delivered ectopically 24 and 48 hours after tumor xenograft at a concentration of 1 µM in serum-free

media. Four days after implantation, tumors were retrieved, fixed in formalin and further subjected to immunohistochemistry.

Immunohistochemistry of CAM and mouse tumors

Formalin-fixed tumors were embedded in paraffin using standard procedures. The 5 µm sections were processed and stained with antibodies directed against PRKD2 (1:250; Abcam #ab51250); pan-cytokeratin (1:80; Dako, clone AE1/AE3); desmin (1:80; Dako, clone D33); von Willebrand Factor VIII (1:100; Biocare Medical, #CP039B); and Ki67 (1:100; Dako, clone MIB-1). Apoptotic cells were detected by TUNEL using the In Situ Cell Death Detection Kit, POD (Roche, # 11684817910) and quantified by counting >700 cells from at least four microscopic fields.

Mouse xenotransplantation experiments

All animal experiments were conducted according to German Animal Welfare and research protocols were approved by the Animal Care and Use Committee at the Regierungspräsidium Tübingen, Germany (TV-1153). MDA-MB231 breast cancer and HCT-116 colon cancer cells (5 x 10^6 each) were subcutaneously inoculated at the left and right dorsal sides of 6 week old female athymic mice (NMRI-(nu/nu), Janvier Labs, Le Genest-Saint-Isle, France. Each experimental group consisting of 9 animals received either 75 mg per kg body weight PU-H71 i.p. three times/week or PBS as vehicle. Tumor size was monitored and measured for the next 3 weeks. After tumor retrieval, tumor volume was calculated according to the formula 0.5xLxWxT (L, length; W, width; T, thickness). Tumors were further processed for immunohistochemistry.

Statistics

Analyses were performed with GraphPad Prims 5.0. Statistical significance was assessed by an unpaired student *t* test. $p<0.05$ was considered significant.

Results

Using an affinity-based proteomic assay followed by chemical precipitation and western blotting validation, Moulick and colleagues (20) identified PRKD2 as a putative HSP90 client in K562 CML cells. In order to assess whether HSP90 is able to bind to PRKD2 in solid tumors, coimmunoprecipitation experiments with pancreatic cancer cells (PaTu2) ectopically expressing GFP-PRKD2 were conducted (**Fig. 1A**). PRKD2 interacted not only with HSP90 but also with the kinase-specific co-chaperone CDC37 (**Fig. 1A**). To investigate whether the stability of PRKD2 requires HSP90, we performed knock-down experiments using short hairpin RNAs (shRNAs) targeting HSP90α (shHSP90α) or HSP90β (shHSP90β) respectively. shRNA-mediated abrogation of both HSP90 isoforms resulted in a decrease of PRKD2 protein levels in lung cancer A549 and breast cancer MDA-MB-231 cell lines (**Fig. 1B** and **1C**) and this was associated with induction of apoptosis as revealed by enhanced PARP cleavage in western blot analysis (**Fig. 1B** and **1C**). These data identify PRKD2 as a novel HSP90 client in epithelial tumor cells and suggest PRKD2 depletion via HSP90 inhibition as a potential strategy to target cancer cells.

HSP90 is highly expressed in many tumors and allows the activation of tumor-specific signaling pathways and buffering stress conditions in the tumor microenvironment (31). Therefore, several ATP-competitive HSP90 inhibitors targeting a wide range of malignant tumors are currently under clinical investigation (1, 20, 32). To investigate whether PRKD2 stability is affected after pharmacologic HSP90 inhibition, eight human cancer cell lines representing six different tumor types (breast cancer, pancreatic cancer, lung cancer, colon cancer, acute myeloid leukemia (AML) and glioblastoma) were incubated for 24 hours with increasing concentrations of two different compounds: PU-H71, an optimized water soluble member of the purine class of HSP90 inhibitors (20) and STA-9090, a resorcinol-containing triazole molecule with a novel chemical structure, both unrelated to the geldanamycin class of HSP90 inhibitors (1). Both inhibitors caused dose-dependent degradation of PRKD2 in all tumor cell lines (**Fig. 1D** and **S.1**). HSP90 inhibition-mediated PRKD2 degradation was associated with increased apoptosis as revealed by augmented PARP and caspase 9 cleavage in all

tumor cell lines (**Fig. 1D** and **S.1**). The enhanced cleaved caspase 9 indicates apoptosis induction via mitochondrial pathway. To prove that HSP90 inhibition-mediated depletion of PRKD2 contributes to the induction of cell death in tumor cells, we first sought to investigate whether down-regulation of PRKD2 preceded the induction of apoptosis. Pancreatic and breast cancer cell lines were incubated with either PU-H71 or STA-9090 for 4, 8, 12, 16, 20 and 24 hours and PRKD2 and cleaved PARP protein levels were assessed by western blotting (**Fig. 1E**). Degradation of PRKD2 commenced after 4 hours (PaTu2) or 8 hours (MDA-MB-231) and was followed by PARP cleavage at around 12 hours in both tumor cell lines, indicating causality between the reduction of PRKD2 protein levels and induction of apoptosis (**Fig. 1E**). To further substantiate that PRKD2 is crucial for killing cancer cells after HSP90 inhibition, we ectopically expressed a GFP-PRKD2 construct in three cancer cell lines (MDA-MB-231, A549 and PaTu2), treated them with PU-H71 and analyzed PRKD2 levels and PARP cleavage by western blotting. Both endogenous and overexpressed PRKD2 were subject of degradation; however the higher remaining PRKD2 protein levels partially rescued cell viability after HSP90 inhibition (**Fig. 2A**). We confirmed these results using a second approach and measured apoptosis by annexin V staining in MDA-MB-231 cancer cells after incubation with PU-H71 for 24 hours. Enforced expression of PRKD2 resulted in near complete rescue of cell viability (**Fig. 2B**). Conversely, overexpression of a kinase-inactive mutant of PRKD2 (GFP-PRKD2-KD) did not prevent cell death triggered by pharmacologic abrogation of HSP90 activity in MDA-MB-231 and PaTu2 cancer cells, suggesting the involvement of PRKD2 kinase activity in tumor cell viability (**Fig. 2C**).

We next characterized the mechanism of PRKD2 degradation after treatment with PU-H71. To assess whether PRKD2 is degraded via the lysosomal pathway, we treated MDA-MB-231 breast cancer and A549 lung cancer cells with the lysosome inhibitor NH_4Cl before PU-H71 incubation. Western blot analysis testing the abundance of PRKD2 protein in the detergent-soluble and detergent-insoluble fractions showed that preincubation with NH_4Cl did not result in an increase of PRKD2 levels compared with the HSP90 inhibitor treatment alone, indicating that PRKD2 is not degraded via the lysosomal pathway (**S.2A**). In contrast, pretreatment of A549 and MDA-MB-231 cell lines with two different proteasome inhibitors, bortezomib or MG-132, followed by incubation with PU-H71 rescued

PRKD2 levels and resulted in redistribution of PRKD2 to the detergent–insoluble fraction (**Fig. 2D** and **S.2B**). Consistent with its degradation via the proteasomal pathway, PRKD2 was extensively ubiquitinated in 293T cells transiently overexpressing PRKD2 after treatment with PU-H71 in combination with bortezomib (**Fig. 2E**). Together, these findings indicated that degradation of PRKD2 upon HSP90 inhibition occurs via the proteasomal pathway.

We previously reported a crucial role for PRKD2 in tumor angiogenesis and cancer cell proliferation (24, 27). We therefore sought to investigate whether PRKD2 depletion via HSP90 inhibition might impair tumor growth and blood vessel formation using a CAM xenotransplantation assay. MDA-MB-231 breast cancer and HCT-116 colon cancer cells stably expressing PRKD2 or empty vector were xenografted on the surface of chicken CAM eight days after egg fertilization. The *in vivo* efficacy of PU-H71 has been previously tested (33). PU-H71 was ectopically applied 24 hours and 48 hours after implantation and tumor growth was monitored. 72 hours after implantation tumors were excised, photographed and analyzed by immunohistochemistry (IHC). Treatment with PU-H71 of cancer cell lines expressing empty vector resulted in a significant decrease in tumor size (**Fig. 3A** and **3B**). IHC analysis showed pronounced PRKD2 degradation upon HSP90 inhibition, which was associated with a significantly reduced proliferation rate as measured by Ki67 staining, and increased apoptosis as determined by TUNEL analysis (**Fig. 3C, 3D, 3E** and **S.3A** and **S.3B**). Examination of tumor-driven vascularization in xenografts revealed a marked reduction of blood vessel density, as determined by desmin and von Willebrand Factor (vWF) staining upon PU-H71 treatment compared to tumors treated with vehicle (**Fig. 3C** and **3F**). Overexpression of PRKD2 was able to revert all PU-H71-induced effects as demonstrated by restored tumor formation (**Fig. 3A** and **3B**), enhanced tumor proliferation (**Fig. 3C**, **3D** and **S.3A**), impaired apoptosis (**Fig. 3C**, **3E** and **S.3B**) and restored blood vessel formation (**Fig. 3C** and **3F**).

To further substantiate the data obtained in the CAM model, we examined the effects of HSP90 inhibition-mediated PRKD2 degradation in an additional *in vivo* model. HCT-116 colon carcinoma and MDA-MB-231 breast cancer cells stably overexpressing PRKD2 or empty vector were injected subcutaneously into both flanks of nude mice. One week later when tumors were palpable, mice

received either 75 mg PU-H71 per kg body weight or vehicle (PBS) i.p. three times per week. After three weeks, mice were sacrificed and tumors were analyzed. In line with the CAM experiments, pharmacologic inhibition of HSP90 resulted in substantially decreased tumor growth, increased apoptosis and impaired angiogenesis in tumors expressing control vector (**Fig. 4A-F, S.4A, S.4B, S.5A** and **S.5B**). Conversely, administration of PU-H71 to mice that received cancer cells overexpressing PRKD2 showed little effect. Tumors from PBS treated mice and tumors overexpressing PRKD2 from PU-H71 treated mice were associated with less TUNEL-positive cells (**Fig. 4D, 4F, S.4A** and **S.4B)**, increased vWF expression (**Fig. 4D** and **4E)** and higher number of Ki67-positive tumor cells as compared to tumors transduced with empty vector and treated with PU-H71 (**S.5A** and **S.5B**). These data are in line with our previous finding that PRKD2 plays a major role in tumor growth and tumor angiogenesis and suggest that these properties can be counteracted by HSP90 inhibition-mediated PRKD2 depletion.

VEGF-A is one of the most potent mediators of the formation of blood vessels both under physiological and pathological conditions. We have previously reported that PRKD2 ablation impairs hypoxia-induced VEGF-A expression and secretion in pancreatic cancer cells (24). Since hypoxic upregulation of VEGF-A occurs mainly via the stabilization of HIF-1α, we sought to investigate whether PRKD2 might regulate VEGF-A via this sensor protein. shRNA-mediated depletion of PRKD2 in pancreatic (PaTu2) and lung (A549) cancer cells abrogated hypoxia-induced accumulation of HIF-1α protein (**Fig. 5A**). In addition, depletion of PRKD2 in tumor cells almost completely prevented the transcriptional activation of the HIF-response element (HRE), a HIF-1α docking site present in promoters that contain the NCGTG sequence (**Fig. 5B**). HIF-1α has been reported to be an HSP90 client (6, 33). In line with this, shRNA-mediated depletion of HSP90 and pharmacologic HSP90 inhibition in breast cancer cells resulted in impaired hypoxia-induced HIF-1α accumulation (**Fig. 5C** and **5D**). In both cases, abrogation of HIF-1α protein stability was associated with decreased hypoxia-induced intracellular VEGF-A levels (**Fig. 5C** and **5D**).

We next considered whether PRKD2 is involved in hypoxia-induced stabilization of HIF-1α during HSP90 inhibition. Therefore, cancer cell lines stably transduced with PRKD2 or empty vector were

incubated in low-oxygen atmosphere in the presence or absence of HSP90 inhibitor. As expected, treatment of tumor cells containing empty vector with PU-H71 impaired hypoxia-stabilized HIF-1α levels (**Fig. 6A** and **6B).** Overexpression of PRKD2 was able to partially rescue the hypoxia-induced accumulation of HIF-1α protein (**Fig. 6A** and **6B**) and HRE promoter activity (**Fig. 6C**), resulting in restored VEGF-A levels secreted by MDA-MB-231 and HCT-116 cells (**Fig. 6D**). Together, these data suggest that hypoxia-induced stabilization of HIF-1α protein is mediated by HSP90 directly and through PRKD2, supporting a concept where PRKD2 links chaperone and hypoxia signaling pathways. VEGF-A can be secreted by tumor cells upon activation of HIF-1α and NF-κB transcription factors (35). Furthermore, low oxygen environment was reported to promote not only the accumulation of HIF-1α but also to activate NF-κB transcription factors via TAK1/IKK signaling (36). HSP90/CDC37 was shown to interact with the kinase domain of IKKα/IKKβ, and inhibition of HSP90 by geldanamycin prevented TNF-induced activation of IKK and NF-κB (37). We therefore wanted to know whether NF-κB signaling might be connected to the hypoxic response regulated by PRKD2 and HSP90. Our experiments show that NF-κB promoter activity is increased upon incubation of MDA-MB-231 cells in low oxygen atmosphere (**Fig. 6E** and **6F**). HSP90 inhibition resulted in impaired hypoxia-induced NF-κB promoter and reduced binding activity (**Fig. 6E** and data not shown). Similarly, shRNA-mediated suppression of PRKD2 resulted in decreased luciferase production of the NF-κB reporter (**Fig. 6F**). We previously demonstrated that hypoxia-induced VEGF-A promoter activity, and intracellular and secreted VEGF-A levels are also impaired upon PRKD2 knock-down in cancer cells (24). Since hypoxia and HSP90 mediate their signals through the IKK complex towards NF-κB which is also activated by PRKD2 (26, 48), we asked whether PRKD2 plays any role in this scenario. A triple active mutant of PRKD2 (PRKD2-S244/706/710E, PRKD2-3SE) was sufficient to enhance the NF-κB promoter activity. Conversely, NF-κB transcriptional activity was impaired upon co-expression of this construct with a non-degradable IκBα mutant (TD-IκBα) (**Fig. 6G**), suggesting that activation of NF-κB by PRKD2 involves the phosphorylation and proteasomal degradation of IκBα. Enforced expression of PRKD2 was able to marginally restore the hypoxia-induced NF-κB

promoter activity affected by the inhibition of HSP90 (**S.6**). Taken together, these results favor PRKD2 as a kinase acting in both NF-κB and HIF-1α pathways thereby integrating hypoxic signals and HSP90 chaperone function to promote tumor growth and tumor angiogenesis.

Discussion

HSP90 serves as an ATP-dependent stabilizer of diverse signaling proteins, including many kinases that are involved in cell proliferation and survival. Chaperone inhibitors were recently shown to effectively inhibit tumor cell growth and angiogenesis in hematologic and solid-organ malignancies. However, it remains elusive whether cancer cell killing or disruption of vasculature network supplying tumor cells is mediated by depletion of a single molecule or simultaneous degradation of multiple client proteins that are overexpressed and/or mutated in cancer (38).

In this study, we have identified PRKD2 as a novel client of the HSP90 chaperone. Besides the interaction with HSP90, we could demonstrate that PRKD2 also interacts with CDC37, a co-chaperone previously reported to recruit protein kinases to the HSP90 chaperone complex (39, 40). Notably, depletion of PRKD2 protein following pharmacologic inhibition of HSP90 dramatically enhanced tumor cell death *in vitro* in various human cancer cell lines, as well as in two *in vivo* xenograft models. These data not only confirm the role of PRKD2 as an anti-apoptotic signaling molecule (41, 42), but also implicate PRKD2 in the cell death evoked by HSP90 inhibition. Our earlier findings demonstrated that PRKD2 is a crucial mediator of tumor angiogenesis involving upregulation and secretion of VEGF-A (24). This prompted us to investigate whether HSP90 might contribute to tumor angiogenesis through PRKD2 protein stabilization. We demonstrated that pharmacologic inhibition of HSP90 impaired blood vessel formation *in vivo*. The fact that PRKD2 overexpression restored vascularization and cell viability after HSP90 inhibition points to the involvement of PRKD2 in these HSP90 inhibitor-induced effects. Our data support PRKD2 degradation through HSP90

inhibition as a putative strategy to hit two important cancer characteristics - angiogenesis and cell viability - with one drug.

HSP90 inhibitors have been reported to indirectly regulate HIF-1α (43 - 46). Furthermore, the HSP90 inhibitor geldanamycin reduced hypoxia-mediated HIF-1α activation, indicating that chaperone activity is needed for this activation (6). The fact that HSP90 interacts both with HIF-1α (6) and PRKD2 (this study) prompted us to evaluate the contribution of PRKD2 with respect to HIF-1α stabilization in hypoxic tumors. We found that abrogation of PRKD2 in cancer cells prevented hypoxia-mediated HIF-1α accumulation and HIF-1α promoter activity. In line with several reports, reduced HSP90 expression and/or activity resulted in impaired hypoxia-triggered HIF-1α accumulation and decreased VEGF-A expression. Notably, ectopically expressed PRKD2 was able to partially restore HIF-1α protein levels, HIF-1α transcriptional activity and secreted VEGF-A levels after pharmacologic HSP90 inhibition. Together, these findings suggest that PRKD2 is required for hypoxia-induced HIF-1α stabilization and that HSP90-supported angiogenesis is modulated by PRKD2 in hypoxic tumors by regulating the accumulation of HIF-1α protein and subsequent VEGF-A secretion.

VEGF-A can be produced by tumor cells upon activation of HIF-1α and NF-κB (35). Hypoxia drives the accumulation of HIF-1α but also activates NF-κB transcription factors (36). Furthermore, inhibition of HSP90 was shown to promote apoptosis through suppression of AKT/NF-κB signaling (47). Thus, NF-κB represents a downstream effector of two major signaling routes: the hypoxia-induced HIF-1α and HSP90 pathways. Since PRKD2 was reported to mediate stress-induced NF-κB activation and cell survival (48), we reasoned that PRKD2 acts upstream of NF-κB and might be a crucial molecule to funnel hypoxia/HIF-1α *and* HSP90 signals down to NF-κB and subsequently to VEGF-A expression/secretion. We found that hypoxia-induced NF-κB activation was blocked by HSP90 inhibition and shRNA-mediated suppression of PRKD2. The finding that PRKD2 was able to just marginally restore hypoxia-induced NF-κB promoter activity affected by the inhibition of HSP90 suggests that other factors might be required as well to funnel HSP90 angiogenic signals through NF-κB pathway.

In conclusion, our data suggest a central role for PRKD2 to enhance HIF-1α accumulation in low-oxygen environment. Stabilization of PRKD2 by HSP90 also results in the activation of NF-κB and its target VEGF-A, which promotes cancer cell growth and increases blood vessel formation in hypoxic tumors (**Fig. 7**). Whether PRKD2 activates NF-κB / VEGF-A via upregulation of HIF-1α in hypoxic tumors or whether it contributes to the parallel activation of distinct HIF-1α / VEGF-A or NF-κB / VEGF-A pathways remains to be elucidated. This study may also have clinical implications since several HSP90 and PRKD2 inhibitors are currently in clinical trials or under development. The combination of HSP90 and PRKD2 inhibitors might have synergistic effects in patients with hypoxic tumors expressing high levels of PRKD2.

Disclosure of Potential Conflicts of Interest

Memorial Sloan-Kettering Cancer Center holds the intellectual rights to PU-H71. Samus Therapeutics, of which G. Chiosis has partial ownership, has licensed PU-H71.

Authors' Contributions

Conception and design: N. Azoitei, T. Seufferlein, C. Scholl, S. Fröhling
Development of methodology: N. Azoitei, K. Diepold, F.Genze, J.Koren III, A. Rouhi, A. Becher, H. Kessler
Acquisition of data: N. Azoitei, K. Diepold, F.Genze, A. Rouhi, C. Brunner
Analysis and interpretation of data: N. Azoitei, A. Rouhi, C.Brunner, G. Chiosis, T. Seufferlein
Writing, review, and/or revision of the manuscript: N. Azoitei, T. Seufferlein, C. Scholl, S. Fröhling, J van Lint, G. Chiosis, C. Brunner
Administrative, technical, or material support: N. Azoitei, T. Seufferlein, G. Chiosis, C. Scholl, S. Fröhling
Study supervision: N. Azoitei, T. Seufferlein

Acknowledgments

The authors thank Susanne Brobovich for excellent technical assistance.

Grant Support

This work was supported by German Research Foundation (DFG; grant AZ.96/1-1) to N. Azoitei, Deutsche Krebshilfe #109373 and DFG SE.676/10-1 to T. Seufferlein and R01 CA172546 and R01 CA155226 grants to G.Chiosis. N. Azoitei and K. Diepold were supported by the DFG grant to N.Azoitei, C. Scholl was supported by an Emmy Noether Fellowship from the DFG.

References

1. Wang Y, Trepel JB, Neckers LM, Giaccone G. STA-9090, a small-molecule HSP90 inhibitor for the potential treatment of cancer. Curr Opin Investig Drugs. 2010 Dec;11(12):1466-76.
2. Semenza G. Signal transduction to hypoxia-inducible factor 1,*Biochem Pharmacol*. 2002 Sep;64 (5-6):993-8.
3. Semenza GL. Regulation of cancer metabolism by hypoxia-inducible factor 1. *Semin Cancer Biol.* 2009 Feb;19(1):12-6. doi: 10.1016/j.semcancer.2008.11.009
4. Hanahan D. Weinberg RA. The hallmarks of cancer. *Cell.*2000; 100:57-70.
5. Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgård R, Tora L, Gassmann M, Poellinger L. Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol Cell Biol.* 1996 Oct;16(10):5221-5231.
6. Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J, Michiels C. Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction. *FEBS Lett.* 1999 Oct 29;460 (2):251-256.
7. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer.* 2005 Oct;5(10):761-72.
8. Caplan AJ, Mandal AK, Theodoraki MA. Molecular chaperones and protein kinase quality control. *Trends Cell Biol.* 2007 Feb;17(2):87-92.
9. Zhao R, Houry WA. Hsp90: a chaperone for protein folding and gene regulation. *Biochem Cell Biol.* 2005 Dec;83(6):703-10.
10. McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M, Frydman J. Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. *Cell.* 2007 Oct 5;131(1):121-135.
11. Picard D. Heat-shock protein 90, a chaperone for folding and regulation. *Cell Mol Life Sci.* 2002 Oct;59(10):1640-1648.
12. Pearl LH. Hsp90 and Cdc37 -- a chaperone cancer conspiracy. *Curr Opin Genet Dev.* 2005 Feb;15(1):55-61.
13. Karnitz LM, Felts SJ. Cdc37 regulation of the kinome: when to hold 'em and when to fold 'em. *Sci STKE.* 2007 May 8;2007(385):pe22.
14. Lee P, Shabbir A, Cardozo C, Caplan AJ. Sti1 and Cdc37 can stabilize Hsp90 in chaperone complexes with a protein kinase. *Mol Biol Cell.* 2004 Apr;15(4):1785-1792.
15. Xu Y, Singer MA, Lindquist S. Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. *Proc Natl Acad Sci* U S A. 1999 Jan 5;96(1):109-14.
16. Basso AD, Solit DB, Chiosis G, Giri B, Tsichlis P, Rosen N. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem.* 2002 Oct 18;277(42):39858-39866.
17. Fujita N, Sato S, Ishida A, Tsuruo T. Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1. *J Biol Chem.* 2002 Mar 22;277(12):10346-10353.
18. Gould CM, Kannan N, Taylor SS, Newton AC. The chaperones Hsp90 and Cdc37 mediate the maturation and stabilization of protein kinase C through a conserved PXXP motif in the C-terminal tail. *J Biol Chem.* 2009 Feb 20;284(8):4921-4935.
19. Zugaza JL, Sinnett-Smith J, Van Lint J, Rozengurt E. Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J.* 1996 Nov 15;15(22):6220-30.
20. Moulick K, Ahn JH, Zong H, Rodina A, Cerchietti L, Gomes DaGama EM, Caldas-Lopes E, Beebe K, Perna F, Hatzi K, Vu LP, Zhao X, Zatorska D, Taldone T, Smith-Jones P, Alpaugh M, Gross SS, Pillarsetty N, Ku T, Lewis JS, Larson SM, Levine R, Erdjument-Bromage H, Guzman ML, Nimer SD, Melnick A, Neckers L, Chiosis G. Affinity-based proteomics reveal cancer-specific networks coordinated by Hsp90. *Nat Chem Biol.* 2011 Sep 25;7(11):818-826.

21. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science.* 2002 Dec 6;298(5600):1912-34.
22. Rykx A, De Kimpe L, Mikhalap S, et al. Protein kinase D: a family affair. *FEBS Lett.* 2003; 546:81-86.
23. Storz P. Mitochondrial ROS eradical detoxification, mediated by protein kinase D. Trends Cell Biol 2007;17:13-18.
24. Azoitei N, Pusapati GV, Kleger A, Möller P, Küfer R, Genze F, Wagner M, van Lint J, Carmeliet P, Adler G, Seufferlein T. Protein kinase D2 is a crucial regulator of tumor cell – endothelial cell communication in gastrointestinal tumors. *Gut.* 2010 Oct;59(10):1316-1330.
25. Kovalevska LM, Yurchenko OV, Shlapatska LM, Berdova GG, Mikhalap SV, Van Lint J, Sidorenko SP. Immunohistochemical studies of protein kinase D (PKD) 2 expression in malignant human lymphomas. *Exp Oncol.* 2006 Sep;28(3):225-230.
26. Mihailovic T, Marx M, Auer A, Van Lint J, Schmid M, Weber C, Seufferlein T. Protein kinase D2 mediates activation of nuclear factor kappaB by Bcr-Abl in Bcr-Abl+ human myeloid leukemia cells. *Cancer Res.* 2004 Dec 15;64(24):8939-44.
27. Azoitei N, Kleger A, Schoo N, Thal DR, Brunner C, Pusapati GV, Filatova A, Genze F, Möller P, Acker T, Kuefer R, Van Lint J, Baust H, Adler G, Seufferlein T. Protein kinase D2 is a novel regulator of glioblastoma growth and tumor formation. *Neuro Oncol.* 2011 Jul;13(7):710-724.
28. Bernhart E, Damm S, Wintersperger A, DeVaney T, Zimmer A, Raynham T, Ireson C, Sattler W. Protein kinase D2 regulates migration and invasion of U87MG glioblastoma cells in vitro. *Exp Cell Res.* 2013 Aug 1;319(13):2037-2048.
29. Wille C, Köhler C, Armacki M, Jamali A, Gössele U, Pfizenmaier K, Seufferlein T, Eiseler T. Protein kinase D2 induces invasion of pancreatic cancer cells by regulating matrix metalloproteinases. *Mol Biol Cell.* 2014 Feb;25(3):324-36
30. Caldas-Lopes E, Cerchietti L, Ahn JH, Clement CC, Robles AI, Rodina A, Moulick K, Taldone T, Gozman A, Guo Y, Wu N, de Stanchina E, White J, Gross SS, Ma Y, Varticovski L, Melnick A, Chiosis G. Hsp90 inhibitor PU-H71, a multimodal inhibitor of malignancy, induces complete responses in triple-negative breast cancer models. Proc Natl Acad Sci U S A. 2009 May 19;106(20):8368-73
31. Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat Rev Mol Cell Biol.* 2010 Jul;11(7):515-28.
32. Whitesell L, Bagatell R, Falsey R. The stress response: implications for the clinical development of hsp90 inhibitors. *Curr Cancer Drug Targets.* 2003 Oct;3(5):349-58.
33. Azoitei N, Hoffmann CM, Ellegast JM, Ball CR, Obermayer K, Gößele U, Koch B, Faber K, Genze F, Schrader M, Kestler HA, Döhner H, Chiosis G, Glimm H, Fröhling S, Scholl C. Targeting of KRAS mutant tumors by HSP90 inhibitors involves degradation of STK33. *J Exp Med*. 2012 Apr 9;209(4):697-711
34. Zhou J, Schmid T, Frank R, Brüne B. PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. *J Biol Chem.* 2004 Apr 2;279(14):13506-13.
35. Ono M. Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. *Cancer Sci.* 2008 Aug;99(8):1501-6
36. Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S. Mechanism of hypoxia-induced NF-kappaB. *Mol Cell Biol.* 2010 Oct;30(20):4901-21
37. Chen G, Cao P, Goeddel DV. TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell.* 2002 Feb;9(2):401-410.
38. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci* U S A. 1994 Aug 30;91(18):8324-8.
39. Siligardi G, Panaretou B, Meyer P, Singh S, Woolfson DN, Piper PW, Pearl LH, Prodromou C. Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. *J Biol Chem.* 2002 Jun 7;277(23):20151-9

40. Gray PJ, Prince T, Cheng J, Stevenson MA, Calderwood SK. Targeting the oncogene and kinome chaperone CDC37. *Nature Reviews Cancer*. 2008 8(7):491-495.
41. Wang QJ, PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol Sci.* 2006 Jun;27(6):317-23.
42. Rozengurt E, Rey O, Waldron RT. Protein kinase D signaling. *J Biol Chem.* 2005 Apr 8;280(14):13205-8.
43. Thomas GV,Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. 2006. *Nat.Med.* 12:122-127.
44. Kim MS, Kwon HJ, LeeYM, et al. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. 2001. *Nat Med.* 7:437-443.
45. Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1a degradative pathway. *J Biol Chem* 2002 277: 29936-29944.
46. Neckers L. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol Med* 2002;8: S55-61.
47. Wang R, Shao F, Liu Z, Zhang J, Wang S, Liu J, Liu H, Chen H, Liu K, Xia M, Wang Y. The Hsp90 inhibitor SNX-2112, induces apoptosis in multidrug resistant K562/ADR cells through suppression of Akt/NF-κB and disruption of mitochondria-dependent pathways. *Chem Biol Interact.* 2013 Sep 5;205(1):1-10
48. Storz P, Toker A. Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. *EMBO J.* 2003 Jan 2;22(1):109-20.

Figure legends

Figure 1. Pharmacologic inhibition of HSP90 results in PRKD2 degradation and induces apoptosis. **A,** immunoprecipitation with anti-GFP antibody was performed with lysates of PaTu2 cells transiently transfected with either GFP-empty vector (e.v.) or a GFP-PRKD2 construct. Membranes were incubated with HSP90α/β and Cdc37 antibodies. **B, C,** protein expression of HSP90α, HSP90β, PRKD2 and cleaved PARP was determined in cancer cell lines transduced with a non-targeting control shRNA (Scr) or a shRNA-targeting HSP90α or HSP90β. **D,** breast cancer (MDA-MB-231), pancreatic cancer (PaTu2), lung cancer (A549), colon cancer HCT-116 and acute myeloid leukemia (AML) cell lines were incubated with increasing amounts of PU-H71 and STA-9090 as indicated. Western blot analysis with PRKD2, cleaved PARP and anti-cleaved caspase 9 are depicted. **E,** lysates of cancer lines incubated with 1μM PU-H71 for the indicated time points were subjected to western blot analysis with PRKD2 and cleaved PARP antibodies.

Figure 2. Destabilization of PRKD2 is essential for HSP90 inhibition-triggered apoptosis in tumor cells. A, lysates of cancer cell lines transiently transfected with empty vector (e.v.) or GFP-PRKD2-widtype (PRKD2-wt) and incubated with PU-H71 for 24 hours were subjected to western blot analysis with PRKD2 and cleaved PARP antibodies. Cleaved PARP bands were quantified by densitometric analysis using ImageJ program (right-hand panels). **B,** cancer cells transiently transfected with empty vector (e.v.) or PRKD2-wildtype (PRKD2-wt) and incubated with PU-H71 for 24 hours were subjected to Annexin V / Propidium iodide staining. The bar graphs represent the means of Annexin V+/PI- cells. **C,** cancer cell lines transfected with a GFP-tagged kinase dead PRKD2 (PRKD2-KD) mutant and incubated with PU-H71 for 24 hours were subjected to western blotting. Membranes were incubated with PRKD2 and cleaved PARP antibodies. Cleaved PARP bands were quantified by densitometric analysis using ImageJ program (right-hand panels). **D,** Soluble and insoluble protein fractions of breast and lung cancer cell lines pre-treated with Bortezomib for 2 h before incubation with PU-H71 were subjected to western blotting. Membranes were incubated with PRKD2 antibody. **E,** 293T cells transfected with GFP-PRKD2 were treated as described in (B) and immunoprecipitation analysis was performed with anti-GFP antibody. Membranes were incubated with anti-Ubiquitin antibody.

Figure 3. HSP90 inhibition impairs tumor growth and tumor blood vessel formation on CAM in a PRKD2-dependent manner A, MDA-MB-231 or HCT-116 stably expressing PRKD2 (PRKD2-wt) or empty vector (e.v.) were delivered to CAM. 24 hours old tumors were treated with 1µM PU-H71 for the next 48 hours. Bar, 1.5 mm. **B,** quantification of tumor area is presented. Error bars represent mean +/- SEM of four to seven tumors. **C,** IHC of HCT-116 cells growing on CAM using specific antibodies directed against PRKD2, Ki67, desmin and von Willebrand factor (vWF) is presented. Parallel samples were subjected to TUNEL labeling. **D,** quantification of Ki67 positive HCT-116 cells is shown. **E,** quantification of TUNEL-positive cells for colon cancer cell xenografted on chicken CAM is displayed. Error bars represent mean +/- SEM of at least four microscopic fields with 700 cells. **F,**

desmin immunoreactivity was quantified by subtracting background staining from specific desmin using Optima software. Scale bar indicates 125 µm.

Figure 4. HSP90 inhibition decreases tumor growth and tumor blood vessel formation in nude mice in a PRKD2-dependent manner. A, PRKD2 expression in colon and breast cancer cell lines stably transduced with PRKD2 is presented. **B,** one week following subcutaneous tumor transplantation mice were injected with PU-H71. Three weeks later animals were sacrificed and tumors were analyzed. Photographs of five representative tumors per experimental group and cell line are depicted. **C,** the volume of explanted tumors was calculated. Graphs represent means +/- SEM of at least 14 tumors per experimental group and cell line as indicated in 4B. **D,** IHC analysis of xenografted HCT-116 tumors with antibodies against von vWF and TUNEL are displayed. **E,** vWF labeling was quantified by subtracting the background staining using Optima software. **F,** quantification of HCT-116 TUNEL-positive tumor cells is presented. Error bars represent mean +/- SEM of at least four microscopic fields with 600 cells. Scale bar indicates 125 µm.

Figure 5. PRKD2 mediates hypoxia-induced accumulation and promoter activity of HIF-1α. A, Pancreatic and lung cancer cell lines stably transduced with PRKD2-specific microRNA (miR-PRKD2) or a non-coding microRNA (miR-Scr) were incubated for 24 hours under low oxygen atmosphere. Cell extracts were subjected to western blot analysis with PRKD2 and HIF-1α antibodies. **B,** breast and lung cancer cell lines transduced with a PRKD2-specific miR were transiently transfected with 3xHRE-luc and pTK-Renilla. Four hours after transfection cells were incubated under normoxic or hypoxic conditions and then cell lysates were subjected to luciferase assay. Bars are the means +/- SEM of at least three independent experiments. **C,** breast cancer cells transduced with a non-targeting control shRNA or HSP90α and HSP90β-specific shRNAs were incubated under hypoxia or normoxia for 24 hours and HIF-1α and VEGF-A levels were determined using western blot analysis. **D,** lysates of breast cancer cells incubated under low oxygen conditions or normoxia in the presence or absence of

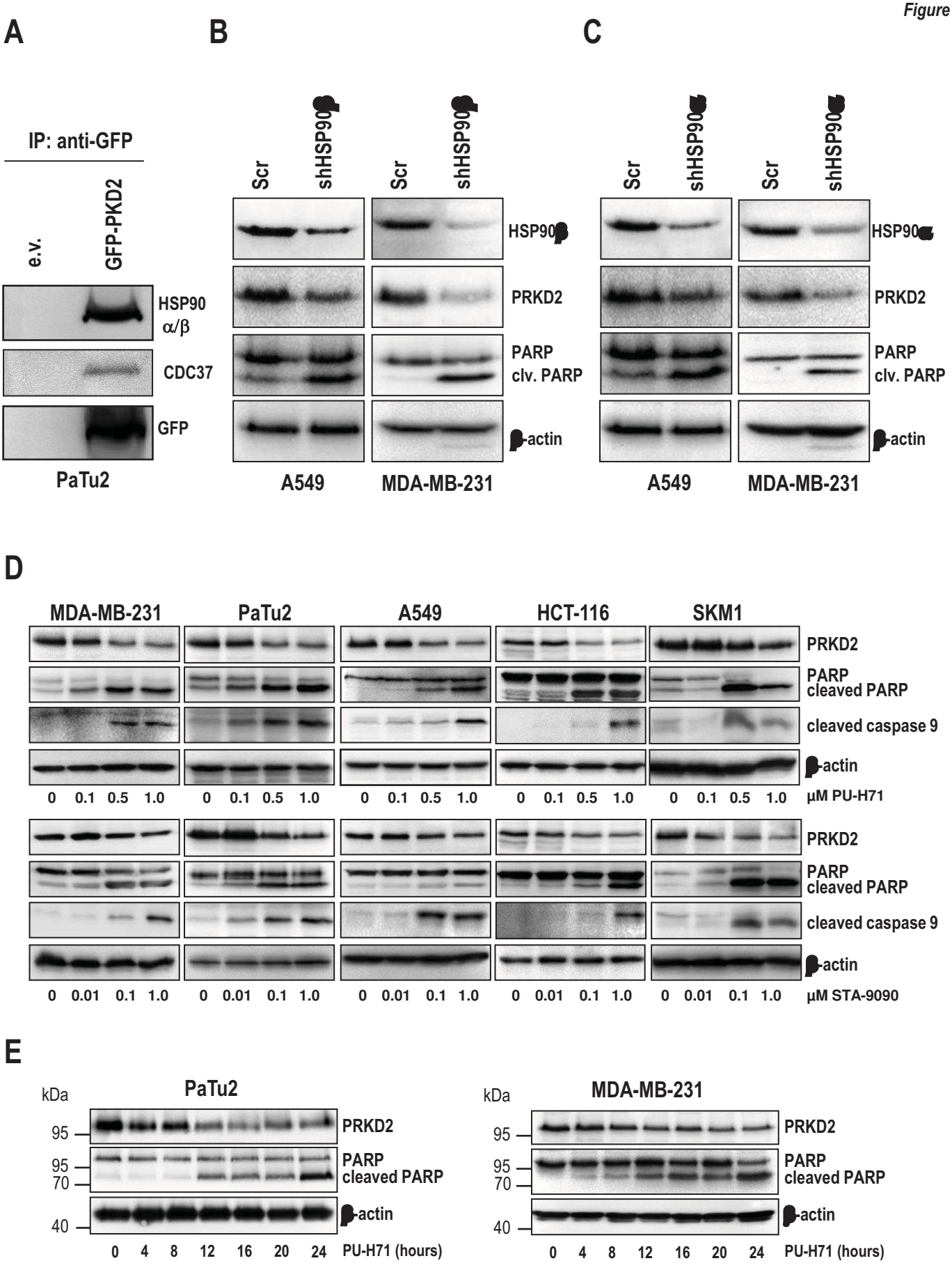
PU-H71 inhibitor were subjected to western blot analysis with HIF-1α and VEGF-A antibodies. No = normoxia; Hy = hypoxia.

Figure 6. Ectopic PRKD2 restores hypoxia-induced stabilization of HIF-1α after HSP90 inhibition. A, B, lung and breast cancer cells transduced with control vector (e.v.) or PRKD2 (PRKD2-wt) were incubated under hypoxic conditions or normoxia for 24 hours in the presence or absence of HSP90 inhibitor. Western blot analysis with HIF-1α and PRKD2 specific antibodies is presented. **C,** breast cancer cells overexpressing PRKD2 were transfected with 3xHRE-luc reporter and then incubated in hypoxic or normoxic environment in the presence or absence of HSP90 inhibitor. Luciferase production was assayed. Bars are the means +/- SEM of three independent experiments. **D,** supernatants of breast and colon cancer cells stably overexpressing PRKD2 or empty vector and incubated in low oxygen in the presence or absence of PU-H71 were subjected to VEGF-A-specific ELISA. **E,** breast cancer cells transfected with 3x κB-luc reporter were incubated in hypoxic or normoxic environment in the presence or absence of HSP90 inhibitor and cell lysates were subjected to luciferase assays. **F,** MDA-MB-231 cancer cells transduced with PRKD2-specific miR transfected with 3x-κB-luc and pTK-Renilla were incubated under normoxic or hypoxic conditions. Cell lysates were subjected to luciferase assay. **G,** Lysates of MDA-MB-231 cells transfected with PRKD2-wt, constitutive active PRKD2 (PRKD2-3SE) and trans-dominant IκBα mutant (TD-IκBα) were subjected to luciferase assay. For all experiments bars represent the means +/- SEM of at least three independent experiments.

Figure 7. PRKD2 modulates HSP90-driven tumor growth and tumor angiogenic by regulating hypoxia-mediated HIF-1α stability and inducing VEGF-A secretion via activation of NF-κB. A, stabilization of PRKD2 by HSP90/Cdc37 chaperone/co-chaperone complex contributes to enhanced HIF-1α accumulation in low oxygen environment. In this scenario, activation of NF-κB and its target VEGF-A is associated with augmented tumor growth and increased blood vessel formation. **B,**

degradation of PRKD2 following HSP90 inhibition affects HIF-1α/VEGF-A and/or HIF-1α/NF-κB/VEGF-A signaling pathways and triggers enhanced cancer cell apoptosis and impaired tumor vascularization. Dotted bold lines represent basal signaling in hypoxic tumors; dotted thin lines represent impaired signaling.

Figure 1


A
IP: anti-GFP
e.v.
GFP-PKD2
HSP90 α/β
CDC37
GFP
PaTu2
B
Scr
shHSP90α/β
HSP90α/β
PRKD2
PARP
clv. PARP
β-actin
A549
MDA-MB-231
C
HSP90α
D
MDA-MB-231
PaTu2
A549
HCT-116
SKM1
PRKD2
PARP
cleaved PARP
cleaved caspase 9
β-actin
0 0.1 0.5 1.0
μM PU-H71
0 0.01 0.1 1.0
μM STA-9090
E
kDa
95
70
40
PU-H71 (hours)
0 4 8 12 16 20 24

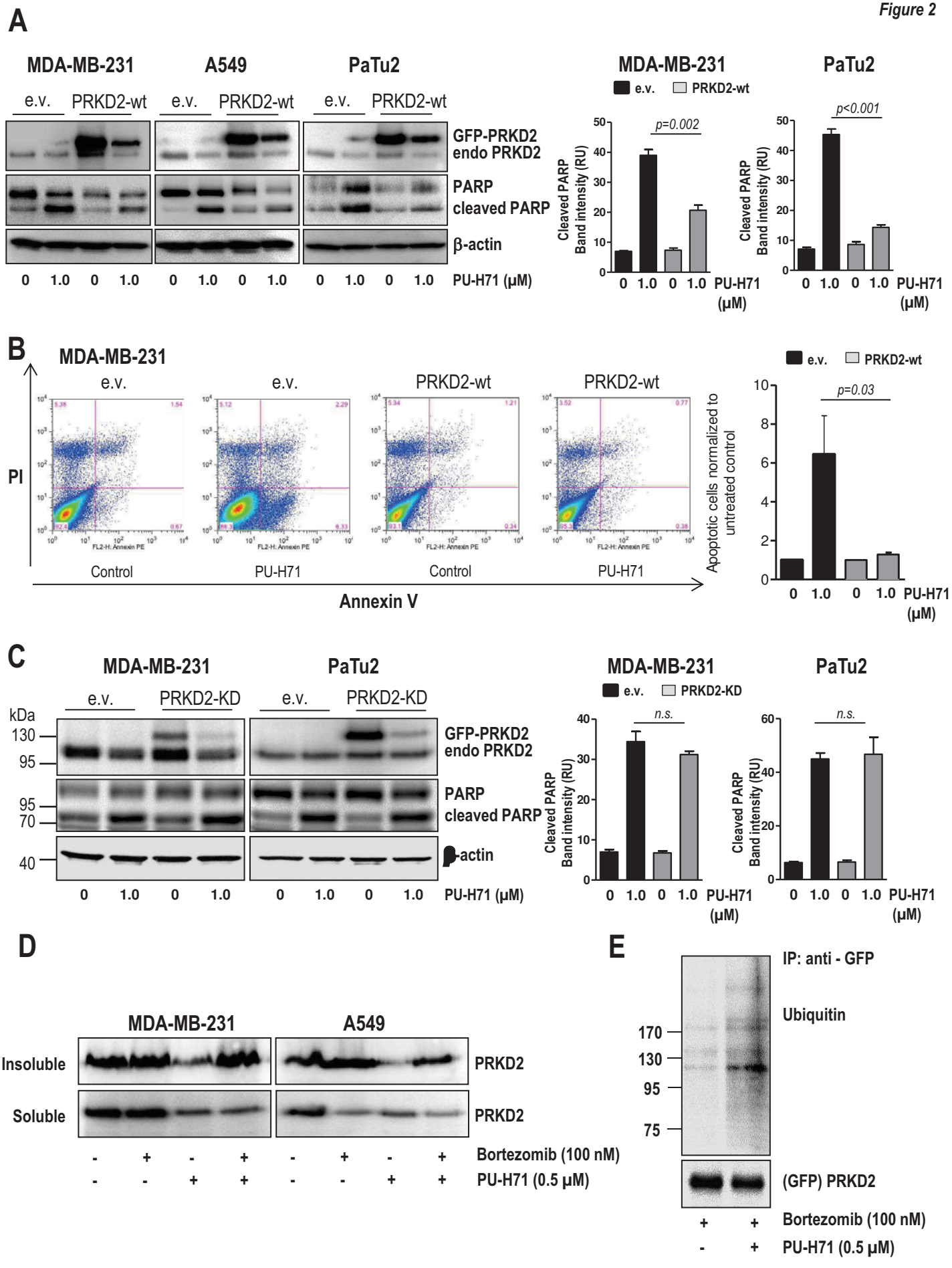
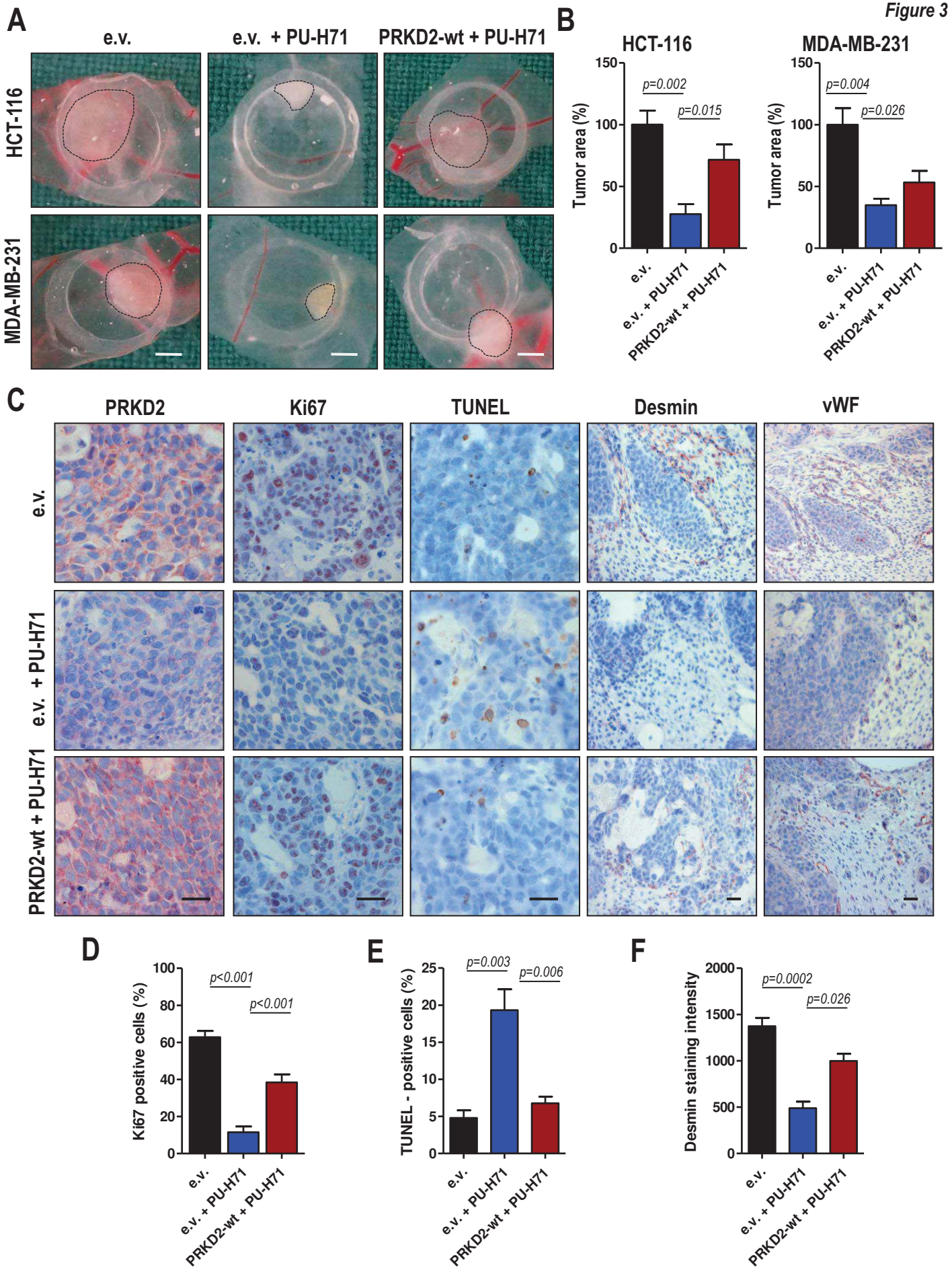
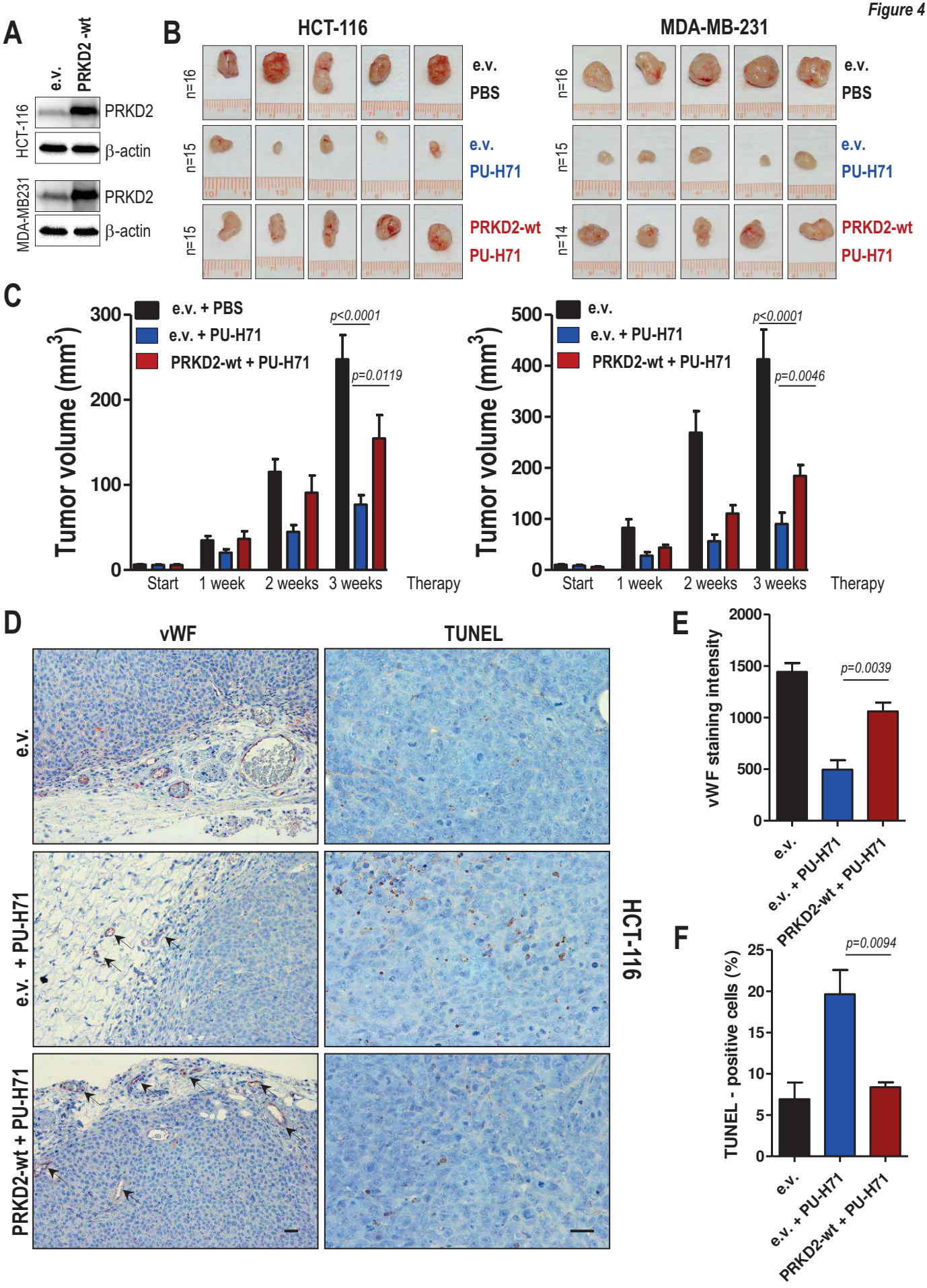
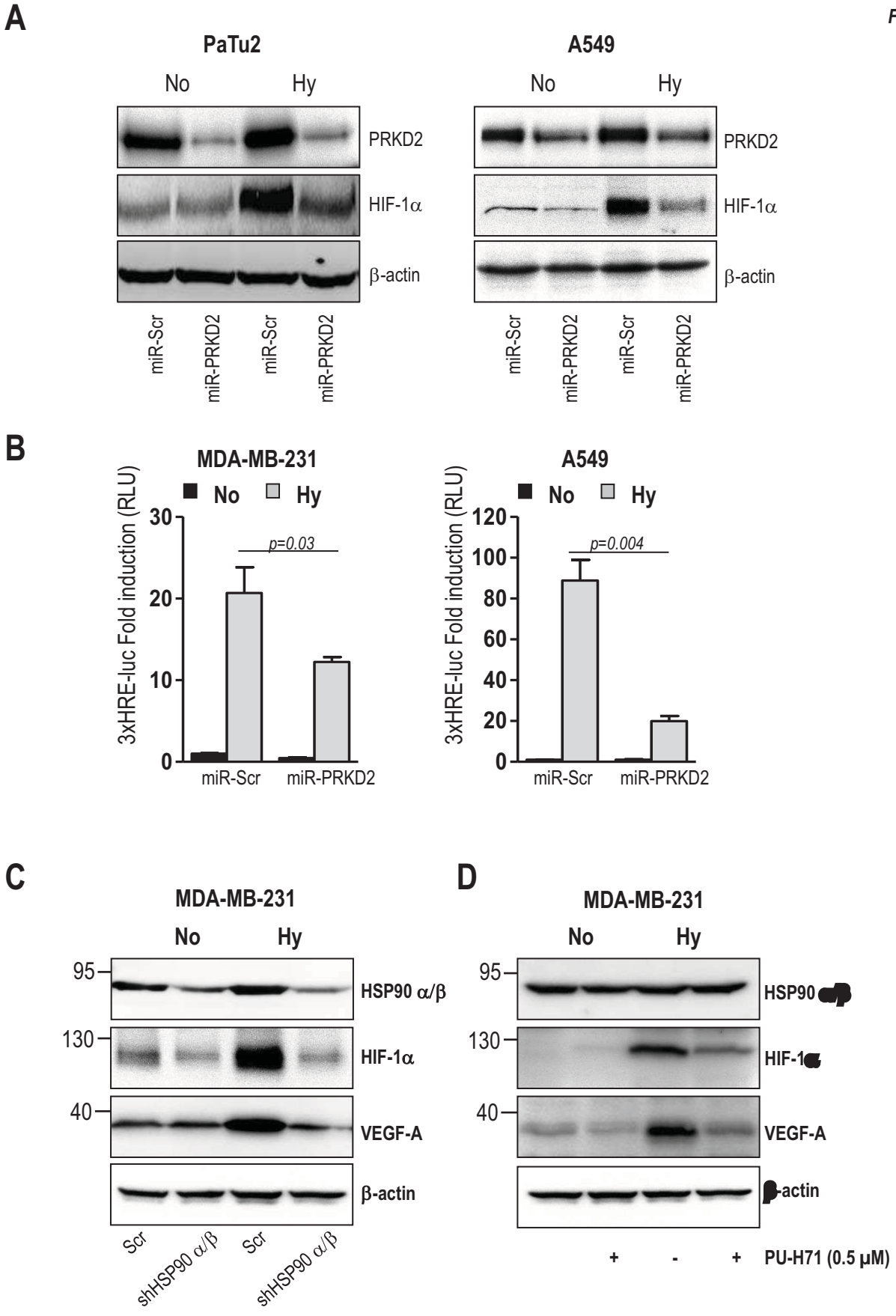
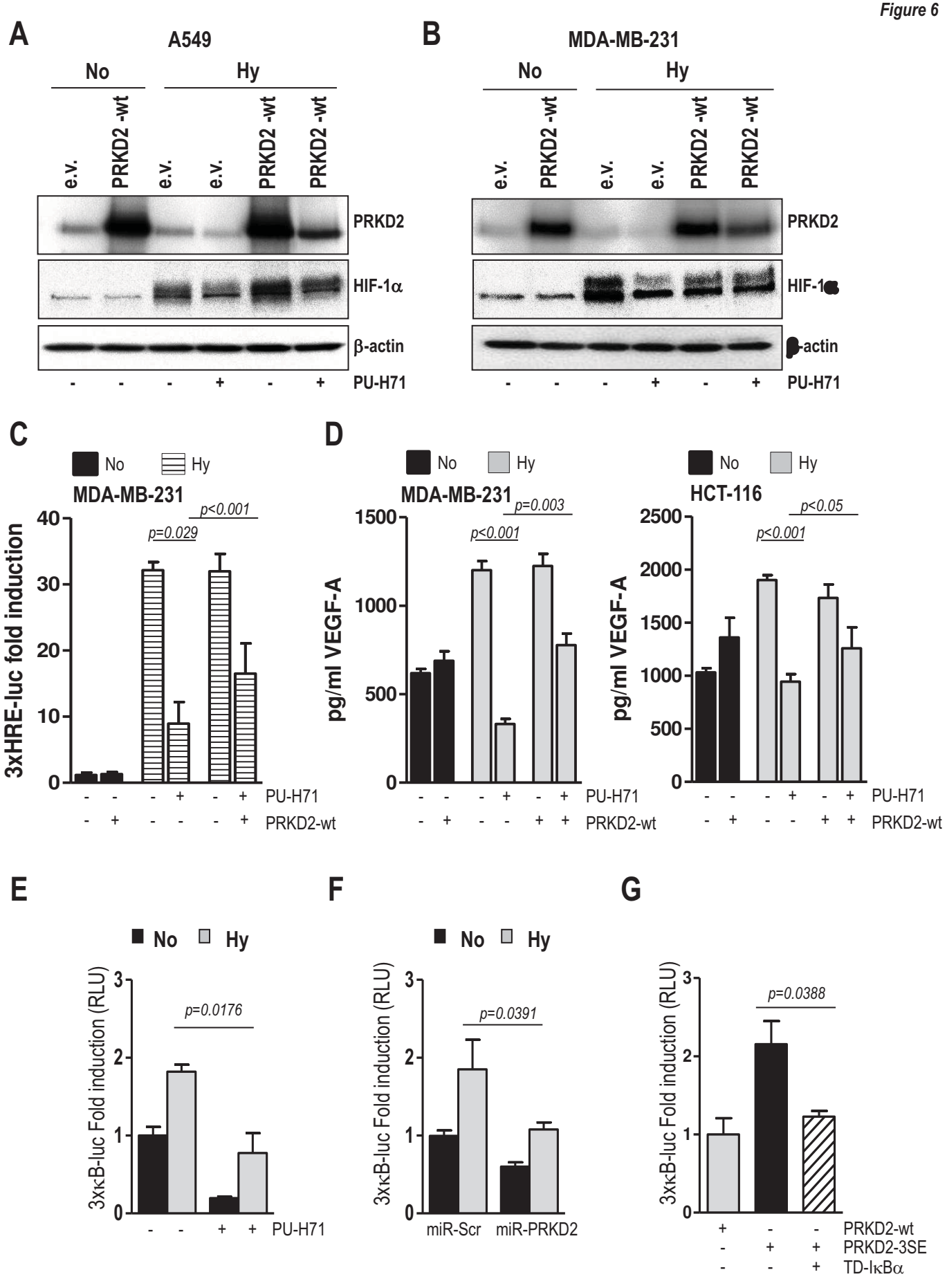

A
MDA-MB-231
A549
PaTu2
e.v.
PRKD2-wt
GFP-PRKD2
endo PRKD2
PARP
cleaved PARP
β-actin
0 1.0 0 1.0 0 1.0 0 1.0 0 1.0 0 1.0 PU-H71 (μM)
MDA-MB-231
PaTu2
e.v.
PRKD2-wt
p=0.002
p<0.001
Cleaved PARP Band intensity (RU)
0 1.0 0 1.0 PU-H71 (μM)
B
MDA-MB-231
e.v.
e.v.
PRKD2-wt
PRKD2-wt
PI
Annexin V
Control
PU-H71
Control
PU-H71
Apoptotic cells normalized to untreated control
p=0.03
0 1.0 0 1.0 PU-H71 (μM)
C
MDA-MB-231
PaTu2
e.v.
PRKD2-KD
kDa
130
95
95
70
40
GFP-PRKD2
endo PRKD2
PARP
cleaved PARP
β-actin
0 1.0 0 1.0 0 1.0 0 1.0 PU-H71 (μM)
MDA-MB-231
PaTu2
e.v.
PRKD2-KD
n.s.
Cleaved PARP Band intensity (RU)
0 1.0 0 1.0 PU-H71 (μM)
D
MDA-MB-231
A549
Insoluble
Soluble
PRKD2
PRKD2
- + - + - + - + Bortezomib (100 nM)
- - + + - - + + PU-H71 (0.5 μM)
E
IP: anti - GFP
Ubiquitin
170
130
95
75
(GFP) PRKD2
+ + Bortezomib (100 nM)
- + PU-H71 (0.5 μM)

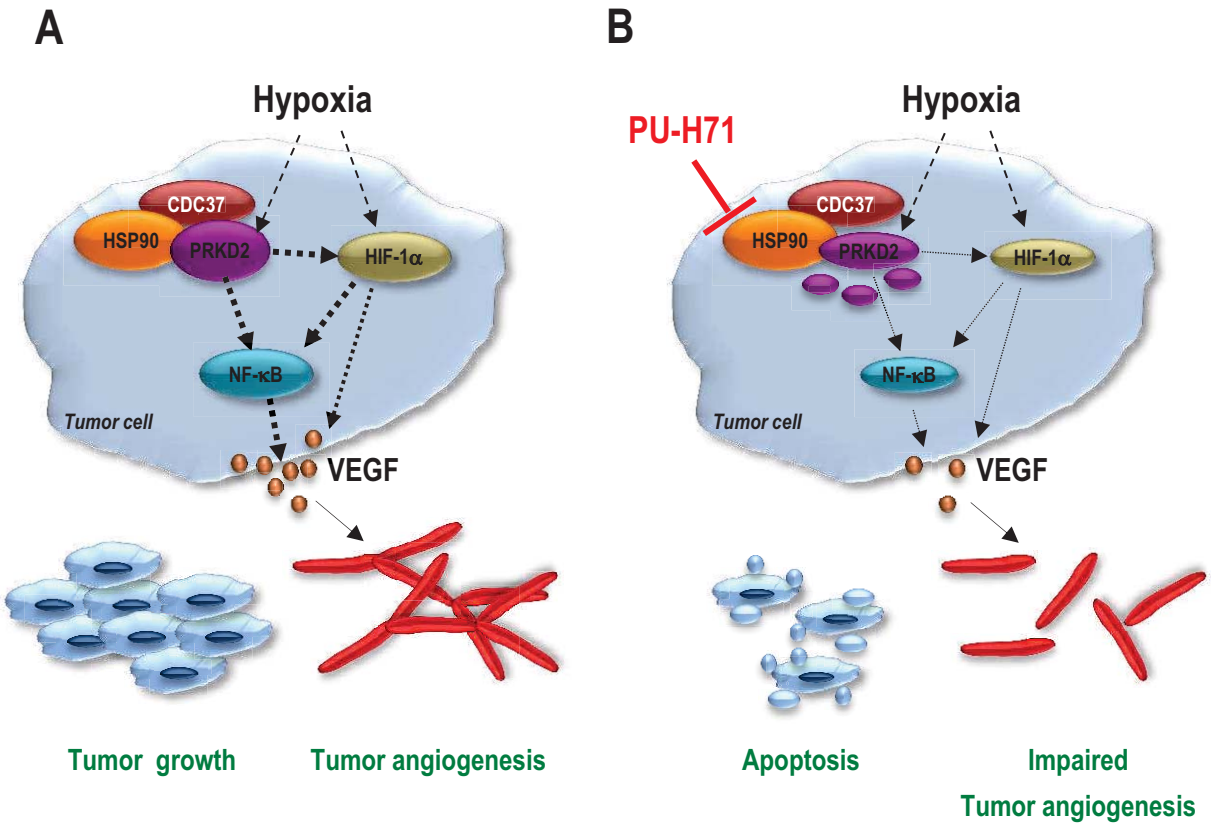
Figure 3


A
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
HCT-116
MDA-MB-231
B
HCT-116
MDA-MB-231
Tumor area (%)
p=0.002
p=0.015
p=0.004
p=0.026
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
C
PRKD2
Ki67
TUNEL
Desmin
vWF
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
D
Ki67 positive cells (%)
p<0.001
p<0.001
E
TUNEL - positive cells (%)
p=0.003
p=0.006
F
Desmin staining intensity
p=0.0002
p=0.026


A
e.v.
PRKD2 -wt
HCT-116
PRKD2
β-actin
MDA-MB231
PRKD2
β-actin
B
HCT-116
MDA-MB-231
n=16
n=15
n=15
n=16
n=15
n=14
e.v.
PBS
e.v.
PU-H71
PRKD2-wt
PU-H71
C
e.v. + PBS
e.v. + PU-H71
PRKD2-wt + PU-H71
Tumor volume (mm3)
p<0.0001
p=0.0119
p=0.0046
Start
1 week
2 weeks
3 weeks
Therapy
D
vWF
TUNEL
e.v.
e.v. + PU-H71
PRKD2-wt + PU-H71
HCT-116
E
vWF staining intensity
p=0.0039
F
TUNEL - positive cells (%)
p=0.0094


A
PaTu2
No
Hy
PRKD2
HIF-1α
β-actin
miR-Scr
miR-PRKD2
miR-Scr
miR-PRKD2
A549
No
Hy
PRKD2
HIF-1α
β-actin
miR-Scr
miR-PRKD2
miR-Scr
miR-PRKD2
B
MDA-MB-231
No
Hy
3xHRE-luc Fold induction (RLU)
30
20
10
0
p=0.03
miR-Scr
miR-PRKD2
A549
No
Hy
3xHRE-luc Fold induction (RLU)
120
100
80
60
40
20
0
p=0.004
miR-Scr
miR-PRKD2
C
MDA-MB-231
No
Hy
95
130
40
HSP90 α/β
HIF-1α
VEGF-A
β-actin
Scr
shHSP90 α/β
Scr
shHSP90 α/β
D
MDA-MB-231
No
Hy
95
130
40
HSP90
HIF-1
VEGF-A
-actin
+
-
+
PU-H71 (0.5 μM)


A
A549
No
Hy
e.v.
PRKD2 -wt
e.v.
e.v.
PRKD2 -wt
PRKD2 -wt
PRKD2
HIF-1α
β-actin
- - - + - + PU-H71
B
MDA-MB-231
No
Hy
e.v.
PRKD2 -wt
e.v.
e.v.
PRKD2 -wt
PRKD2 -wt
PRKD2
HIF-1α
β-actin
- - - + - + PU-H71
C
No
Hy
MDA-MB-231
3xHRE-luc fold induction
40
30
20
10
0
p<0.001
p=0.029
- - - + - + PU-H71
- + - - - + PRKD2-wt
D
No
Hy
MDA-MB-231
pg/ml VEGF-A
1500
1000
500
0
p=0.003
p<0.001
- - - + - + PU-H71
- + - - + + PRKD2-wt
No
Hy
HCT-116
pg/ml VEGF-A
2500
2000
1500
1000
500
0
p<0.05
p<0.001
- - - + - + PU-H71
- + - - + + PRKD2-wt
E
No
Hy
3xκB-luc Fold induction (RLU)
3
2
1
0
p=0.0176
- - + + PU-H71
F
No
Hy
3xκB-luc Fold induction (RLU)
3
2
1
0
p=0.0391
miR-Scr
miR-PRKD2
G
3xκB-luc Fold induction (RLU)
3
2
1
0
p=0.0388
+ - - PRKD2-wt
- + + PRKD2-3SE
- - + TD-IκBα


A
Hypoxia
CDC37
HSP90
PRKD2
HIF-1α
NF-κB
Tumor cell
VEGF
Tumor growth
Tumor angiogenesis
B
Hypoxia
PU-H71
CDC37
HSP90
PRKD2
HIF-1α
NF-κB
Tumor cell
VEGF
Apoptosis
Impaired
Tumor angiogenesis